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Sexual dimorphism in the spontaneous recovery from spinal cord injury: a gender gap in beneficial autoimmunity?

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Abstract

Immune cells have been shown to contribute to spontaneous recovery from central nervous system (CNS) injury. Here we show that adult female rats and mice recover significantly better than their male littermates from incomplete spinal cord injury (ISCI). This sexual dimorphism is wiped out and recovery is worse in adult mice deprived of mature T cells. After spinal cord contusion in adult rats, functional recovery (measured by locomotor scores in an open field) was significantly worse in females treated with dihydrotestosterone prior to the injury than in placebo-treated controls, and significantly better in castrated males than in their noncastrated male littermates. Post-traumatic administration of the testosterone receptor antagonist flutamide promoted the functional recovery in adult male rats. These results, in line with the known inhibitory effect of testosterone on cell-mediated immunity, suggest that androgen-mediated immunosuppression plays a role in ISCI-related immune dysfunction and can therefore partly explain the worse outcome of ISCI in males than in female. We suggest that females, which are more prone to develop autoimmune response than males, benefit from this response in cases of CNS insults.

Introduction

Traumatic spinal cord injury occurs most commonly in young men; approximately 60% of patients are men aged 16–30, and only one patient in five is a female (National SCI Statistical Center, http://www.spinalcord.uab.edu) (Schwab & Bartholdi, 1996). In most cases patients sustain an incomplete spinal cord injury (ISCI), which often results in greater functional deficits than might be expected from the severity of the initial damage. The additional loss is due to a process of secondary degeneration, largely caused by an injury-induced toxic excess of certain physiological compounds. Recent studies in our laboratory have shown that the injured nerve can benefit from the activity of T cells directed against myelin-associated self-antigens (Moalem *et al.*, 1999a; Hauben *et al.*, 2000a). In rats and mice, passive transfer of such autoimmune T cells immediately after axonal injury to the central nervous system (CNS) resulted in a reduction of the post-traumatic neuronal losses both in the partially injured optic nerve and in the contused spinal cord (Hauben *et al.*, 2000b; Moalem *et al.*, 1999a; Moalem *et al.*, 1999b; Hauben *et al.*, 2000a; Moalem *et al.*, 2000a; Moalem *et al.*, 2000b). Our group further demonstrated that this protective autoimmunity is a physiological cell-mediated response, which is spontaneously evoked in certain individuals and strains and is amenable to boosting by active immunization with the appropriate CNS antigens (Hauben *et al.*, 2001a; Hauben *et al.*, 2001b; Kipnis *et al.*, 2001; Yoles *et al.*, 2001). Whether spontaneously induced or exogenously boosted, this systemic autoimmune response, provided that it is suitably regulated, can lead to an improvement in the outcome of a CNS injury (Hauben *et al.*, 2001a; Jones *et al.*, 2002). Taken together, these results suggest that the injury-evoked spontaneous T cell-mediated immune response plays a critical role in both the maintenance of undamaged axons and the repair of partially damaged ones, leading to improved spontaneous recovery from ISCI.

The intensity of the immune response is known to differ between men and women. Immune responses tend to be more vigorous in females, resulting in production of more antibodies and increased cell-mediated immunity after vaccination (Lahita, 1997). Autoimmune diseases are more prevalent in women than in men (Whitacre *et al.*, 1999), suggesting that gonadal hormones such as oestrogens, progesterone, and testosterone may modulate the susceptibility to these disorders (Paavonen *et al.*, 1981; Weinstein *et al.*, 1984; Sthoeger *et al.*, 1988). As early as 1898, Calzolari reported that the thymus of male rabbits castrated before sexual maturity was larger than that of noncastrated males (Calzolari, 1898; Staples *et al.*, 1999). In mice, allograft rejection occurs earlier in females and in castrated males than in normal males (Graff *et al.*, 1969). Estrogens stimulate the activities of macrophages and lymphocytes (Offner *et al.*, 2000; Bebo *et al.*, 2001), and peritoneal macrophages harvested from female rats release larger amounts of interleukin (IL)-1 than those from age-matched males (Friedman *et al.*, 1985; Hu *et al.*, 1988). Thymocytes and lymphocytes from normal female mice respond more vigorously to exogenous and allogeneic antigens than do cells from male mice (Weinstein *et al.*, 1984). Testosterone ameliorates experimental autoimmune encephalomyelitis (EAE) through a direct effect on T lymphocytes (Dalal *et al.*, 1997; Bebo *et al.*, 1998; Benten *et al.*, 1999; Liva & Voskuhl, 2001).

In the United States, more than 75% of patients suffering from multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus

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(SLE), and thyroiditis are women (Jacobson *et al.*, 1997). It was shown that changing the sex steroid environment could alter the onset and course of autoimmune SLE in the F1 NZB/NZW mouse model. Female mice of this strain normally develop SLE, but prepubertal administration of dihydrotestosterone (DHT) prevents development of the disease (Roubinian *et al.*, 1979). In addition, castration of males leads to SLE development (Michalski *et al.*, 1983). In castrated F1 males administration of androgens has a protective effect, which is eliminated by thymectomy (Sheridan, 1991). The notion that male and female sex steroids differentially affect autoimmune processes is further supported by the finding that in women with SLE, androgens are present in smaller amounts and active oestrogen metabolites in larger amounts than in age-matched healthy female controls. Furthermore, oestrogenic activity was found to be increased in SLE patients (Lahita *et al.*, 1982; Lahita, 1997).

Immune responses to trauma also exhibit sexual dimorphism (for review see Angele *et al.*, 2000). A linkage, however, between sexual differences in recovery from CNS insult and immune activity has never been explored. Cellular immune responses after trauma are depressed in males, but unchanged or enhanced in females (Wichmann *et al.*, 1996a). Following a traumatic event (haemorrhage), the splenic and peritoneal macrophage immune responses and the release of Th1 lymphokines (IL-2, interferon-γ) by splenocytes is enhanced in proestrous mice, in contrast to a depressed immune response and increased release of IL-10 in males (Wichmann *et al.*, 1996a; Wichmann *et al.*, 1997b; Kahlke *et al.*, 2000a; Kahlke *et al.*, 2000b; Kahlke *et al.*, 2000c).

The purpose of the present work was to determine, using rats and mice with injured spinal cords as models, whether the ability to manifest a protective T cell-mediated immune response after ISCI (and hence the functional outcome of the injury) displays sexual dimorphism, and if so, whether it is related to androgens.

Materials and methods

Animals

Inbred adult Lewis or Sprague–Dawley rats (10–12 weeks old, 200–250 g) and normal and nude Balb/c mice were supplied by the Animal Breeding Center of The Weizmann Institute of Science. Rats were housed in a light- and temperature-controlled room and were matched for age in each experiment. All animals were handled according to NIH guidelines for the management of laboratory animals.

Spinal cord injury

Rats were anaesthetized by intraperitoneal injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and their spinal cords were exposed by laminectomy at the level of T8. One hour after induction of anaesthesia, we dropped a 10-g rod onto the laminectomized cord from a height of 50 mm (severe injury) or 25 mm (mild injury), using the NYU impactor, a device shown to inflict a well-calibrated contusive injury of the spinal cord (Basso *et al.*, 1996; Young, 1996; Hauben *et al.*, 2000a; Hauben *et al.*, 2000b).

Mice were anaesthetized as described above and their spinal cords were exposed by laminectomy at the level of T12. To stabilize the spinal cord we applied adjustable forceps to the spinous processes of two vertebrae, the one proximal and the other distal to the laminectomy site. Using the NYU impactor we then placed a 10-g rod (2 mm diameter) on the exposed cord for 2 min, according to Farooque (Farooque, 2000), with some modifications. The rod was then removed and the skin sutured.

Castration

To examine the effect of testosterone on recovery from ISCI, we used male Sprague–Dawley rats and Balb/c mice aged 12 weeks. Animals were anaesthetized as described above. The scrotum was incised transversely and the testicles were exposed, ligated and removed. The incision was closed with a single skin clip. In sham-operated rats and mice the scrotum was incised and sutured, but the testicles were not removed. After a recovery period of 14 days, castrated males and their sham-operated littermates underwent spinal cord trauma.

Dihydrotestosterone administration

Ten days before spinal cord contusion, adult female Sprague–Dawley rats were anaesthetized as described above and a 21-day-release pellet containing 100 mg DHT (Innovative Research of America, Sarasota, FL) or a placebo pellet containing the vehicle was inserted subcutaneously into their necks.

Flutamide treatment

The testosterone inhibitor flutamide, 25 mg/kg body weight (Sigma-Aldrich, Israel) or an equal volume (0.3 mL) of the nontoxic vehicle propanediol (Wichmann *et al.*, 1997a; Ba *et al.*, 2001; Messingham *et al.*, 2001) was injected intraperitoneally (i.p) into adult male Sprague–Dawley rats immediately after spinal cord contusion. Thereafter, the rats were injected i.p. with flutamide (5 mg/kg body weight, treated group) or an equivalent volume of the vehicle (control group) every second day until the 10th day after injury.

Animal care

In spinally injured rats and mice, bladder expression was assisted by massage at least twice a day (particularly during the first 48 h after injury, when it was carried out up to three times a day), until the end of the second week, by which time automatic voidance had been recovered. Animals were carefully monitored for evidence of urinary tract infection or any other sign of systemic disease. During the first week after contusion and in any case of haematuria after this period, they received a course of sulfamethoxazole (400 mg/mL) and trimethoprim (8 mg/mL) (Resprim, Teva Laboratories, Israel), administered orally with a tuberculin syringe (0.3 mL of solution per day). Daily inspections included examination of the laminectomy site for evidence of infection and assessment of the hind limbs for signs of autophagia or pressure.

Assessment of recovery from spinal cord contusion

Behavioural (functional) recovery was operationally defined by locomotor activity, which was determined by locomotor hindlimb performance. This was scored using the open-field locomotor rating scale of Basso, Beattie, and Bresnahan (BBB), on a scale of 0 (complete paralysis) to 21 (normal mobility) (Basso *et al.*, 1996; Young, 1996; Hauben *et al.*, 2000b; Jakeman *et al.*, 2000; Hauben, 2001a; Ma *et al.*, 2001). Blind scoring ensured that observers were not aware of the treatment received by each rat or mouse. Approximately once a week we evaluated the locomotor activities of the trunk, tail, and hind limbs in an open field by placing the animal for 4 min in the centre of a circular enclosure (90 cm diameter, 7 cm wall height) made of molded plastic with a smooth, nonslip floor. Prior to each evaluation the rat or mouse was examined carefully for perineal infection, wounds in the hind limbs, and tail and foot autophagia.

Inclined plane test

The neurological function of spinally contused male and female Sprague–Dawley rats from the experiment described in Fig. 1A was also assessed, using the inclined plane test of Rivlin and Tator (Rivlin & Tator, 1977). In this test the rat is placed on a rectangular board, which is raised through increments of five degrees from the horizontal. The inclined plane score represents the maximum inclination at which the rat can maintain itself for 5 s.

Retrograde labelling of rubrospinal neurons

This procedure was performed as previously described (Hauben *et al.*, 2000b). In brief, three months after spinal cord contusion we re-anaesthetized three rats from each group and applied the dye rhodamine dextran amine (Fluoro-ruby, Molecular Probes Eugene, OR) below the site of contusion at T12. After five days the rats were deeply anaesthetized again and their brains were excised, processed, and cryosectioned. Sections containing the red nucleus were inspected and analyzed qualitatively and quantitatively by fluorescence and confocal microscopy (Midha *et al.*, 1987; Theriault & Tator, 1994). Only animals with some intact rubrospinal fibers are capable of supporting their own weight (Fehlings & Tator, 1995).

Histology

Four months after undergoing severe spinal cord contusion, two male and two female Sprague–Dawley rats were perfused intracardially with 100 mL of cold phosphate-buffered saline (PBS), followed by 200 mL of paraformaldehyde (4% prepared in 0.1 M phosphate buffer with glucose 5%). Their spinal cords were removed, postfixed overnight in 10% paraformaldehyde, rinsed briefly in PBS, and embedded in a paraffin block. Serial sections (4 µm) from each block were stained with haematoxylin and eosin or Luxol Fast Blue.

Statistical analysis

Behavioural and morphological data were analyzed by two-tailed Student *t*-tests. As the open-field motor scores were measured at different time points after the injury, they were also analyzed by two-factor repeated-measures ANOVA.

Results

Sexual dimorphism in recovery from spinal cord contusion

Rats were subjected to a severe spinal contusion. We made sure that males and females were matched for weight and age (in each experiments), so that the severity of the primary loss due to the mechanical insult would be identical in all animals in each experiment. Any differences in recovery would then reflect the extent of secondary loss. Assessment of recovery was based on locomotion in an open field measured by the BBB locomotor rating scale (Basso *et al.*, 1996), in which a value of 0 represents complete paralysis and a value of 21 represents complete mobility. Spontaneous recovery from spinal cord contusion in adult male and female rats ($n = 5$ in each group) was examined first in Lewis rats. After severe spinal cord contusion, the hindlimb motor performance was significantly better in females than in males. Motor scores differed significantly with time ($P < 0.01$) and gender (two-factor repeated-measures ANOVA; $d.f. = 1$, $F = 24.887$, $P < 0.005$). A two-tailed *t*-test disclosed significant differences, starting from day 27 after the injury and at every time point tested thereafter (Fig. 1). The scores (mean ± SEM) during the 14th and 15th weeks after contusion were 6.5 ± 0.18 for females and 3.3 ± 0.47 for males ($P < 0.001$).

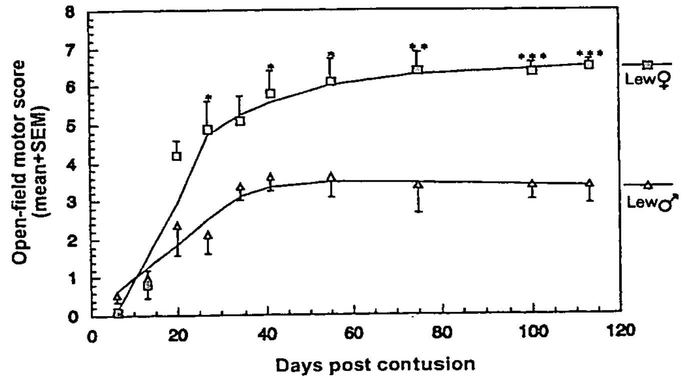

FIG. 1. Sexual dimorphism in spontaneous recovery from incomplete spinal cord injury (ICSI). Five female and five male Lewis rats were deeply anaesthetized and subjected to severe spinal cord contusion at T8 (NYU impactor, 10 g, 50 mm). Recovery was assessed by the BBB open-field locomotor rating scale at the indicated time points. Results are expressed as mean values for each group (error bars indicate SEM). The differences were significant ($P < 0.005$, two-factor repeated-measures ANOVA; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed *t*-test).

A similar experiment was performed with Sprague–Dawley rats subjected to severe (Fig. 2A) or mild (Fig. 2B) spinal contusion. Here too, the functional recovery of females was significantly better than that of males (two-factor repeated-measures ANOVA $d.f. = 1$, $F = 20.847$, $P < 0.0005$ and $d.f. = 1$, $F = 16.516$, $P < 0.005$, respectively).

In another experiment rats were subjected to severe spinal contusion, and when their BBB scores reached a plateau each rat was examined for its ability to maintain itself for 5 s on an inclined plane. The highest slope at which the rats could maintain themselves for 5 s was 31.0 ± 1.0 degrees for the males and 42.3 ± 1.9 degrees for the females ($P < 0.001$, two-tailed *t*-test; Table 1). Maximal BBB values of these rats were 2.9 ± 0.6 (mean ± SEM) in the males ($n = 5$) and 6.4 ± 1.1 in the females ($n = 4$) ($P < 0.01$, two-tailed *t*-test; Table 1).

To determine whether the difference in functional recovery between males and females is related to postinjury survival of their supraspinal tracts, we retrogradely labelled the rubrospinal tracts by labelling the red nuclei (Fig. 3). As intact rubrospinal tracts (indicated by retrograde labelling) are found only in animals capable of at least partial weight support (Fehlings & Tator, 1995; Hauben *et al.*, 2000b), such labelling yields an all-or-none indication of weight support depending on whether the rat's BBB score is above or below the weight-support threshold. Thus, rats with BBB values lower than 8 are likely to show little or no retrograde labelling of rubrospinal neurons in the red nuclei. We examined three male rats with BBB scores of 5.0, 5.0, and 5.5 (mean ± SEM, 5.3 ± 0.2) and three females with BBB scores of 8.5, 9.5, and 8.0 (8.7 ± 0.4) of the groups described in Fig. 2B. The mean scores and mean numbers of rubrospinal neurons are recorded in Table 2. For both parameters, the differences between male and female rats were significant.

No sexual dimorphism in the spontaneous recovery of nude mice from spinal cord injury

To determine whether the observed gender-related differences in recovery can be attributed to an ability to exhibit a T cell-mediated immune response to injury, we compared the functional recovery of male and female wild-type (WT) mice to that of male and female

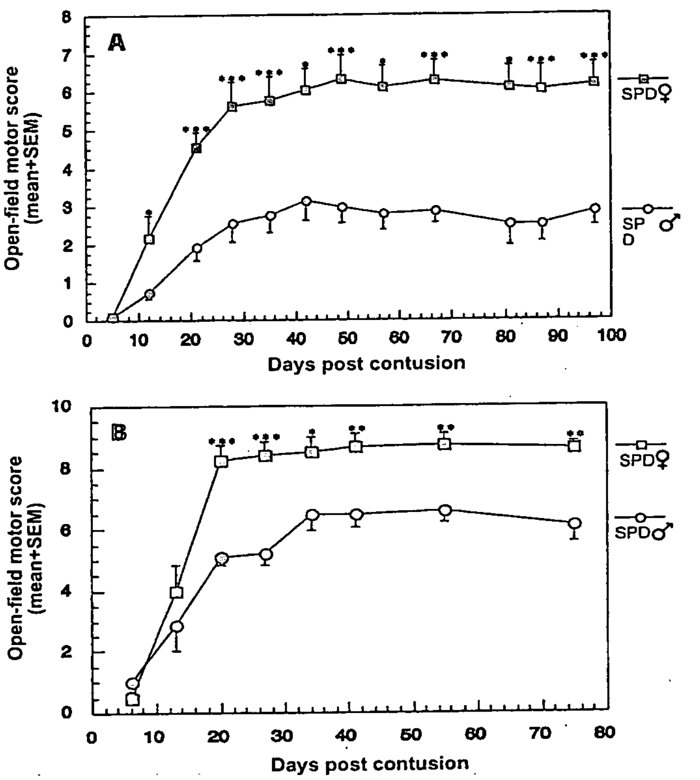

FIG. 2. Sexual dimorphism is evident in the response to injuries of different severity. (A) Nine female and 11 male SPD rats were deeply anaesthetized and subjected to severe spinal cord contusion at T8 (NYU impactor, 10 g, 50 mm). Recovery was assessed by the BBB open-field locomotor rating scale at the indicated time points. B. Six female and five male SPD rats were subjected to mild spinal cord contusion (NYU impactor, 10 g, 25 mm). Results are expressed as the mean values for each group (error bars indicate SEM). The differences were significant (A: P <0.0005, B: P <0.005, two-factor repeated-measures ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$, two-tailed *t*-test).

TABLE 1. Comparison of motor scores determined by the BBB locomotor rating scale and the inclined plane test

	Females (n = 4)	Males (n = 5)	P-value*
Maximal BBB score	6.4 ± 1.1	2.9 ± 0.6	< 0.01
Maximal slope (degrees)	42.3 ± 1.9	31 ± 1.0	< 0.001

SPD rats were subjected to severe spinal contusion by a weight drop (10 g) from a height of 50 mm, using the NYU impactor. After reaching a plateau in locomotor performance, rats were scored for their ability to maintain themselves on an inclined plane. Results are presented as means ± SEM. P-values and statistical difference notations represent sex difference effects.
*Two-tailed Student's *t*-test.

mice devoid of mature T cells, i.e. nude (nu/nμ) mice. For this purpose we selected a mouse strain (Balb/c) that is capable of exhibiting a spontaneous T cell-dependent beneficial response to a CNS insult (Kipnis *et al.*, 2001). Male and female WT and nude Balb/c mice were subjected to ISCI (see Methods) and their functional recovery was evaluated according to the BBB locomotor rating scale. As expected, WT females (n = 6) recovered significantly better than

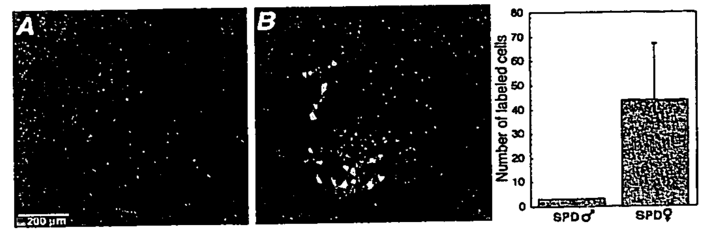

FIG. 3. A representative photomicrograph of red nuclei from one male and one female Sprague–Dawley rat of the groups described in Fig. 2A. After severe spinal cord contusion, hardly any rubrospinal neurons were observed in the male. The representative slices shown here were taken from a male rat with a BBB score of 2.5 and a female rat with a BBB score of 7 (graph shows mean + SEM; n = 3).

TABLE 2. Relationship between the recovery rate measured by the BBB open field locomotor rating scale and the preservation of rubrospinal tracts measured by retrograde labelling of red nuclei neurons

	Females (n = 3)	Males (n = 3)	P-value*
Maximal BBB score	8.7 ± 0.4	5.3 ± 0.2	< 0.005
No. of labelled neurons	61.3 ± 3.8	1 ± 1	< 0.005

SPD rats were subjected to mild spinal cord contusion by a weight drop (10 g) from a height of 25 mm, using the NYU impactor. After a plateau was reached in their locomotor performance (see Fig. 2B), the numbers of spared rubrospinal neurons in their red nuclei were retrogradely labelled and then counted. Results are presented as means ± SEM.
*Two-tailed Student's *t*-test.

WT males (n = 5) ($d.f.$ = 1, F = 5.148, $P < 0.05$, two-factor repeated-measures ANOVA). No differences were detected between females and males when nude mice were used. Moreover, in both genders recovery of the nude mice was worse than that of the WT mice, as shown by the significantly greater loss of function after contusion in nude mice than in WT mice (two-way repeated-measures ANOVA; $d.f.$ = 1, F = 15.504, $P < 0.01$ in males and $d.f.$ = 1, F = 15.541, $P < 0.005$ in females; Fig. 4).

Castration improves functional recovery from spinal cord injury in rats and mice

The lack of sexual differences in functional outcome in the nude mice suggested that the positive effect of oestrogen or the negative effect of androgen on the recovery of mice from ISCI is T cell-dependent. Testosterone plays a role in male post-traumatic immunosuppression, and gonadal hormones were shown to affect cell-mediated immune responses (Angele *et al.*, 1998; Angele *et al.*, 2000). This testosterone-induced immunosuppressive effect might occur in spite of a transient reduction observed in testosterone levels after injury (Huang *et al.*, 1995). It was therefore of interest to determine whether the poor recovery of males from ISCI might be related to androgen-mediated immunosuppression. Six Balb/c mice were anaesthetized and castrated, and eight control littermates were anaesthetized and sham-operated. Two weeks later the mice were subjected to spinal cord injury at the level of T12. Motor performance was evaluated weekly using the BBB locomotor rating scale. Starting on day 13 and at all times of examination thereafter, the castrated mice showed significantly better locomotor performance than their sham-operated littermates (Fig. 5A). Thus, 3 months after compression injury the BBB score was 11.2 ± 0.5 in the castrated males and

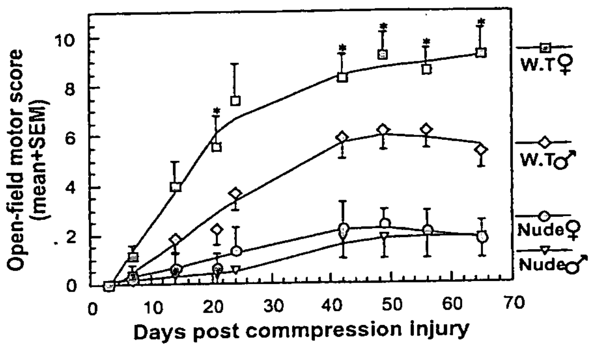

FIG. 4. Recovery from spinal cord compression in male and female Balb/c mice compared to their matched nude counterparts. Spinal cord compression at T12 (10 g, 2 min) was inflicted on mice in four groups (WT females, $n = 6$, WT males, $n = 5$; nude males, $n = 4$, nude females, $n = 4$. Two-factor repeated-measures ANOVA: WT males compared with WT females, $P < 0.05$; WT males compared with nude males, $P < 0.001$; WT females compared with nude females, $P < 0.005$; nude females compared with nude males, no difference. Two-tailed t-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The results suggest that functional recovery in nude mice is significantly worse than in WT mice, and that nude mice do not show sexual dimorphism in their spontaneous recovery from ISCI.

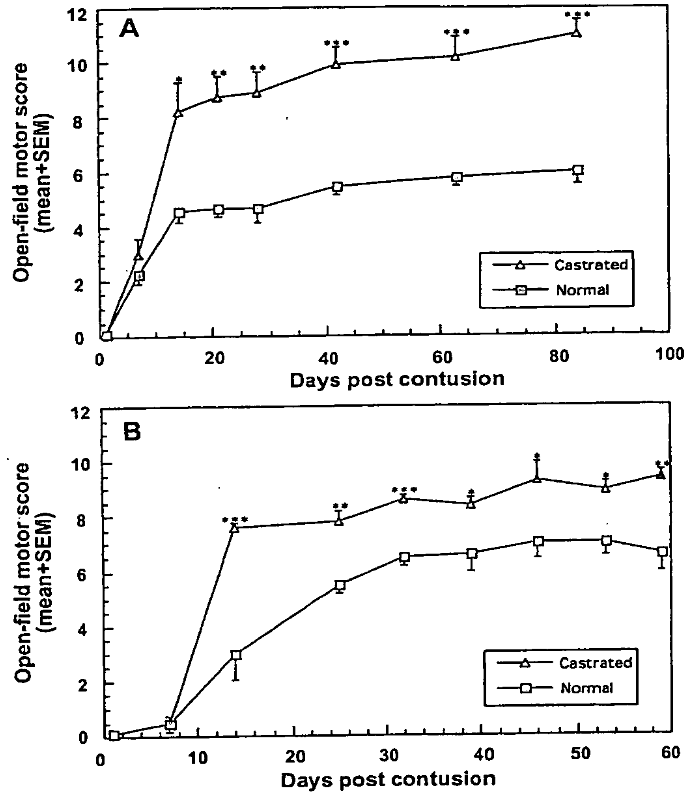

FIG. 5. Castration improves spontaneous functional recovery from spinal cord injury. (A) Fourteen days after castration, castrated ($n = 6$) and intact ($n = 8$) male Balb/c mice were subjected to ICSI. Hindlimb locomotion was assessed weekly with the BBB locomotor rating scale. Castrated mice performed significantly better than intact mice ($P < 0.0005$, two-factor repeated-measures ANOVA). (B) The pattern was similar when castrated and intact Sprague–Dawley rats ($n = 5$ per group) were subjected to a mild contusive injury ($P < 0.005$, two-factor repeated-measures ANOVA; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed t-test).

5.8 ± 0.3 in the controls (two-factor repeated measures ANOVA; $d.f. = 1$, $F = 23.31$, $P < 0.0005$). A similar pattern was observed in Sprague–Dawley male rats that were anaesthetized, castrated or sham-operated ($n = 5$ per group), and subjected 14 days later to mild spinal cord contusion (Fig. 5B; 9 ± 0.2 in castrated males and 6.5 ± 0.3 in controls, two-factor repeated-measures ANOVA; $d.f. = 1$, $F = 19.665$, $P < 0.005$). These findings suggest that the sexual dimorphism observed in functional recovery from ISCI may, at least in part, be androgen dependent.

Testosterone interferes with recovery from spinal cord injury

To further substantiate the connection between androgens and recovery from ISCI, female Sprague–Dawley rats were anaesthetized and a DHT or placebo pellet (21-day-release, 100 mg) was inserted subcutaneously in the neck of each rat. Ten days later these rats were subjected to severe spinal cord contusion. Figure 6A and B record the open-field motor scores of the two groups ($n = 5$ in each group). DHT had a significantly detrimental effect on functional recovery from ISCI (two-factor repeated-measures ANOVA; $d.f. = 1$, $F = 6.234$, $P < 0.05$).

Figure 6C and D record the open-field motor scores of male Sprague–Dawley rats treated with flutamide, a nonsteroidal antagonist of the testosterone receptor. Flutamide (25 mg/kg, i.p) was administered immediately after severe spinal cord contusion, and thereafter every second day (5 mg/kg, i.p) until the 10th day after the contusion, as described in Materials and methods. Control males were treated with the nontoxic vehicle propanediol according to the same administration protocol. Rats treated with flutamide recovered significantly better than the vehicle-treated controls (two-factor repeated-measures ANOVA; $d.f. = 1$, $F = 5.284$, $P < 0.05$). These findings suggest that testosterone has a negative effect on the spontaneous recovery from ISCI.

Morphological analysis of the lesion site in male and female rats subjected to identical primary insults

Four months after the rats were subjected to severe spinal cord contusion, the spinal cords of two male and two female Sprague–Dawley rats were sectioned longitudinally (4 μm), stained with haematoxylin and eosin or Luxol Fast Blue (Fig. 7), and examined by phase-contrast microscopy. The spinal cords of the male rats showed severe loss of neural tissue and large cyst-like structures (Fig. 7C and D). Damage was also evident in the spinal cords of females (Fig. 7A and B), but there was more preservation of neural tissue, better tissue organization, and greater continuity of myelinated fibers than in the males. These findings support the contention that the better functional recovery observed in females may be attributable to improved tissue preservation, possibly as a result of endogenous neuroprotective processes that do not occur in the male.

Discussion

The results of this study suggest that the better spontaneous recovery of female rats and mice after ISCI than that of males is related to the suppressive effect of androgens on the ability to sustain a T cell-mediated protective response to a CNS insult. It should be noted that immune cell-mediated neuroprotection refers to an endogenous mechanism of protection that is recruited by the individual to resist the consequences of CNS insults. Such a mechanism slows down injury-induced processes of degeneration, leading to the rescue of

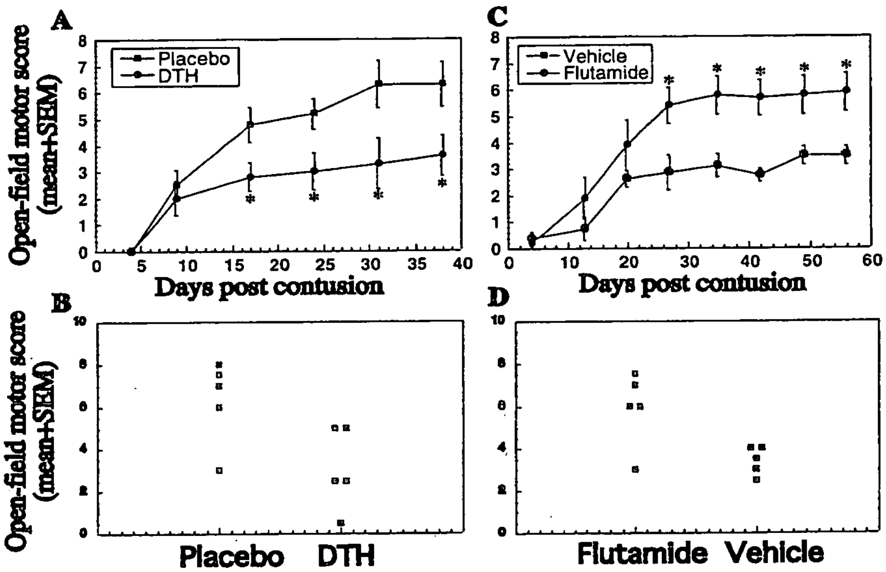

FIG. 6. Detrimental effect of testosterone on spontaneous recovery from ISCI. (A and B) Five Sprague–Dawley female rats were treated subcutaneously with DHT pellets (21-day-release, 100 mg) and five were treated with placebo pellets containing the vehicle only. Ten days later the rats were subjected to severe spinal cord contusion. (A) The spontaneous recovery of female rats treated with DHT was significantly worse than that of placebo-treated females (*$P < 0.05$, two-tailed t-test; $P < 0.05$, two-factor repeated-measures ANOVA). Results are expressed as means ± SEM of the locomotion score on the indicated days following the contusion. (B) Open-field motor scores of these individual female rats 38 days after injury. (C and D) Post-traumatic i.p. administration of the testosterone receptor antagonist flutamide in male Sprague–Dawley rats ($n = 5$ in each group) resulted in significant improvement in their functional recovery from spinal cord contusion. (C) Mean open-field motor scores of flutamide-treated and vehicle-treated male rats ($n = 5$, *$P < 0.05$, two-tailed t-test; $P < 0.05$, two-factor repeated-measures ANOVA). (D) Open-field motor scores of these individual male rats 56 days after the injury.

neurons that escaped the primary insult (Moalem *et al.*, 1999a; Hauben *et al.*, 2000b) but in the absence of neuroprotection would eventually undergo secondary degeneration (Agrawal & Fehlings, 1996; Lynch & Dawson, 1994; Yoles & Schwartz, 1998). The physiological protective mechanism varies in efficacy according to the individual's genetic background. For therapeutic purposes it is often insufficient and sometimes severely limited; however, it is amenable to boosting (Hauben *et al.*, 2000b; Kipnis *et al.*, 2001; Yoles *et al.*, 2001).

Over the last few years our research group has formulated the concept of 'protective autoimmunity' (Schwartz *et al.*, 1999, 2001), which implies that a benign T cell-mediated immune response, evoked by a CNS insult and directed to self-antigens residing in the site of the lesion, helps the body to overcome the effects of destructive self-compounds that emerge from the injured tissues. These antiself (autoimmune) T cells home to the site of injury, where they become activated upon encountering their specific antigens presented to them by antigen-presenting cells (Schwartz & Kipnis, 2001). Recent studies from our laboratory have shown that autoimmune responsible for neuroprotection following injury of myelinated axons are Th1 cells (Kipnis *et al.*, 2002a), which are directed against myelin antigens (Kipnis *et al.*, 2002b). In EAE-susceptible strains these cells are potentially encephalitogenic. Because females are known to have both a greater ability to sustain an autoimmune response and a higher incidence of autoimmune diseases than males (Whitacre *et al.*, 1999), it was of interest to determine whether females would show better or worse recovery than males from ISCI. In other words, we were interested in finding out whether spinally injured female rats and mice can benefit from their inherent ability to sustain a more vigorous immune response to injury-related self-antigens than that in males. If so, this might be interpreted as a certain advantage of a stronger self-reactive immunity under traumatic conditions.

Clinical experience, as well as a substantial body of documented evidence, suggests that females recover better than males from various types of traumatic injury (Angele *et al.*, 2000; Kahlke *et al.*, 2000a; Roof & Hall, 2000; Stein, 2001). Our finding that the relative advantage of female mice in their spontaneous recovery from ISCI is diminished in the absence of mature T cells suggests that this advantage is related to the ability to spontaneously manifest a T cell-mediated protective immune response (Kipnis *et al.*, 2001; Yoles *et al.*, 2001). It thus appears that females, because of their enhanced T cell-mediated immunity, can recover from traumatic injury better than males, but that this advantage comes at the cost of a greater susceptibility to autoimmune diseases in certain individuals and strains. In both susceptible and resistant strains, however, the females recover from ISCI better than the males. Males may possess a testosterone-related mechanism that directly or indirectly decreases their susceptibility to autoimmunity (Roubinian *et al.*, 1979; Michalski *et al.*, 1983; Bebo *et al.*, 1998; Olsen & Kovacs, 2001). It is therefore possible that in the course of evolution, males and females adapted ways that led to the development of gender-specific strategies to cope with the dichotomy represented by the concomitant existence of protective and destructive autoimmunity.

In the behavioural assessment of the effects of sex hormones, 'organizational' (long-lasting perinatal or prepubertal) effects must be distinguished from 'activational' (postpubertal temporary) effects. Behavioural (Williams *et al.*, 1990), neural (Bimonte *et al.*, 2000) and immune (Olsen *et al.*, 2001) systems display sexual dimorphisms that include both organizational and activational components. The greater capacity of females than males to spontaneously manifest a postinjury neuroprotective autoimmune response might derive from the sug-

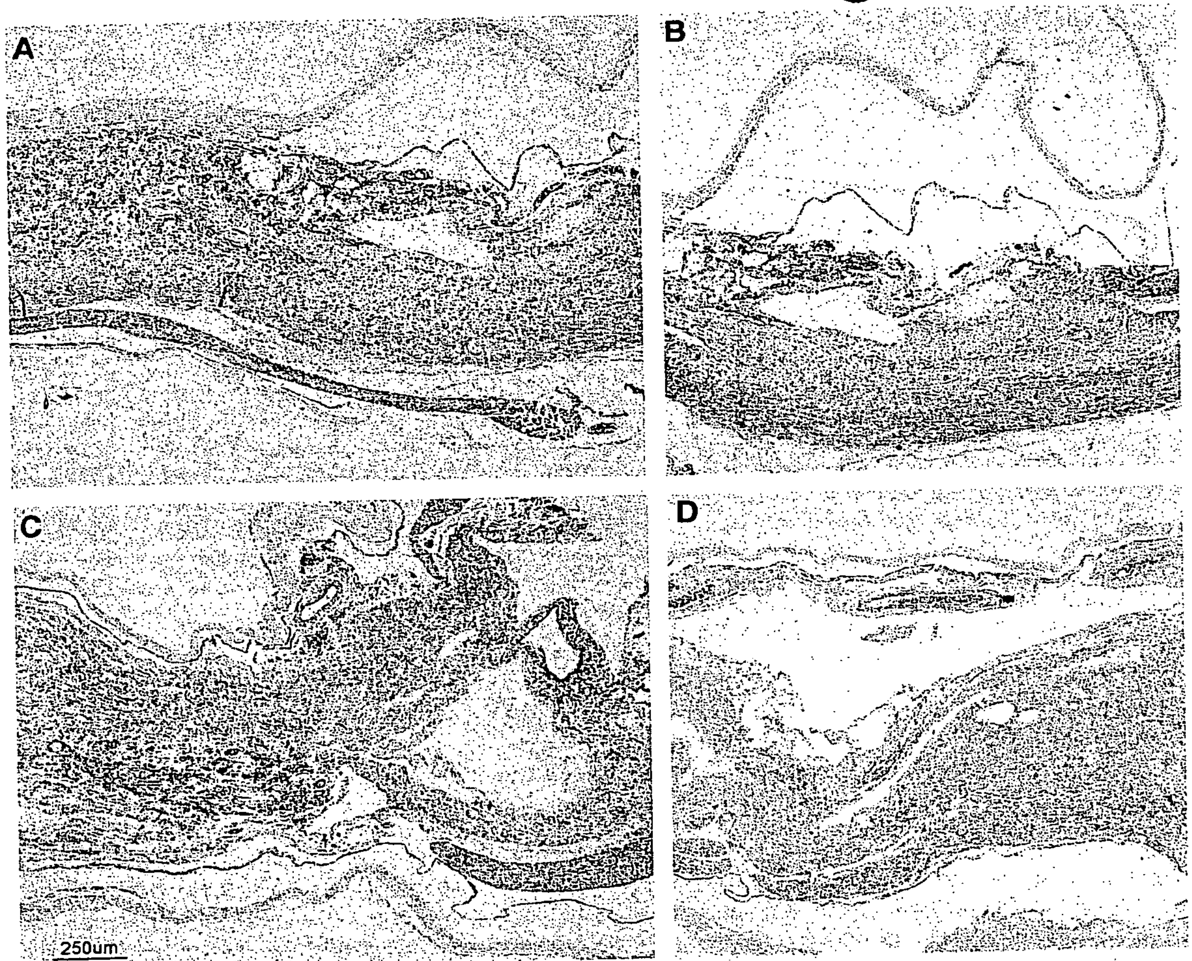

FIG. 7. Histological comparison of spinal cords of male and female Sprague–Dawley rats subjected to identical insults. Four months after severe spinal contusion, longitudinal sections (4 μm) were taken from the contused spinal cords of female (A and B) and male (C and D) Sprague–Dawley rats and stained with haematoxylin and eosin (A and C) or Luxol Fast Blue (B and D). Note the high degree of neural tissue organization (A) and the continuous white matter staining (B) in the female, in contrast to the loss of structural organization and myelinated fibers in the male.

gested ability of female sex hormones to directly or indirectly evoke immune activity (Wichmann *et al.*, 1996a; Kammer & Tsokos, 1998; Huber *et al.*, 1999a; Huber *et al.*, 1999b; Offner, 2000). It might also be an outcome of the ability of male hormones to suppress such activity (Angele *et al.*, 1998; Dalal *et al.*, 1997; Wichmann *et al.*, 1997b; Liva & Voskuhl, 2001). Testosterone regulates many physiological and behavioural processes, including the activation of male sexual behaviour, brain sexual differentiation, and negative feedback effects of steroid hormones on gonadotropin secretion (Balthazart & Ball, 1998). Our findings suggest that sexual dimorphism in the recovery from traumatic injury results, at least in part, from an androgen-related suppressive effect on the immune system.

In the present study, all treated animals were adults and therefore the effects could all be defined as activational. However, recovery from CNS injury involves processes of neural tissue reorganization, including degenerative and regenerative processes, which may have permanent consequences on the morphology and function of the CNS (McDonald & Sadowsky, 2002). Protective treatments applied within the therapeutic time window (approximately 2 weeks) can significantly improve the outcome, whereas later interventions are ineffective (Hauben *et al.*, 2000b; Zeman *et al.*, 2001). Thus, after ISCI the spinal tissue apparently undergoes a phase during which sex hormones and immune factors can modify both functional and anatomical properties and exert long-lasting effects on tissue reorganization. In the present study, treatment of adult female rats with 21-day-release DHT pellets administered 10 days prior to ISCI had long-lasting negative locomotor effects that persisted for at least 6 months after the injury (not shown). In addition, the improved locomotor performance observed in adult males treated with the testosterone receptor antagonist flutamide lasted long after the treatment had ended and the functional and morphological differences between male and female rats persisted for at least 120 days post injury (Figs 1 and 7). Because activational effects, by definition, are likely to be seen only when the hormone is present, we suggest that the long-lasting effect of testosterone is, at least in part, organizational.

The observed differences in recovery between males and females subjected to standardized primary mechanical insults reflect different extents of secondary loss, which are affected by the ability to spontaneously manifest protective immunity (Yoles *et al.*, 2001). Behavioural assessment of Sprague–Dawley rats in the present study disclosed that the recovered locomotor activity, measured by the BBB locomotor rating scale (see Materials and methods), in some females

subjected to severe spinal cord contusion exceeded the threshold value of 8 (i.e. the rats show some degree of weight support), and was significantly higher than in males. The superior functional recovery of these female rats was confirmed by retrograde labelling of the rubrospinal neurons (Figs 2A and 3) and (in an additional experiment) by the inclined plane test, which revealed significant gender-related differences (Table 1). Incline plane scores were previously shown to correlate with corticospinal neuronal survival (Fehlings & Tator, 1995). The relationship between the BBB values and the numbers of surviving rubrospinal neurons in the red nuclei in rats subjected to mild spinal cord contusion (Table 2) further supported the notion that the gender-related differences result not from differences in behaviour but from anatomical differences in the numbers of spared neurons in the central corticospinal pathways (Hauben *et al.*, 2000b).

Spinal cord injury leads to changes in the function of immune cells and alterations in immune functions such as the release of inflammatory cytokines (Cruse *et al.*, 1996; Segal *et al.*, 1997; Pan *et al.*, 2002). Our observation that nude mice recover significantly less well than wild-type mice from ISCI is in agreement with previous findings by our group and by others (Schori *et al.*, 2001; Serpe *et al.*, 1999). This observation, substantiates the role of certain immune functions, and specifically of T lymphocytes, in the promotion of functional recovery from ISCI (Hauben *et al.*, 2000a; Hauben *et al.*, 2000b; Bethea & Dietrich, 2002; David & Ousman, 2002). In the present study, sexual dimorphism was observed in the spontaneous recovery from ISCI in wild-type, but not in nude mice. This observation confirms the suggestion of other authors that sexual dimorphism in the recovery from trauma is related, at least in part, to the participation of immune functions in recovery from traumatic injury (Angele *et al.*, 2000; Kovacs *et al.*, 2002). The improved recovery of females from ISCI may be attributed to oestrogens (Roof, 2000; Behl, 2002), and especially to the high production of 17-β-estradiol (E2), shown to directly affect the maintenance of immune functions after traumatic haemorrhage (Knoferl *et al.*, 2002). The role of oestrogens in the induction of sexual dimorphism and in recovery from trauma is currently under investigation (Gregory *et al.*, 2000; Knoferl *et al.*, 2000; Knoferl *et al.*, 2001; Stein, 2001).

Our findings do not exclude the possibility of a direct effect of sex hormones, through their specific receptors in the spinal cord (Vito *et al.*, 1979; MacLusky *et al.*, 1987), on recovery from ISCI. Moreover, there is evidence for a synergistic role of androgens and oestrogens in promoting peripheral nerve regeneration in a model of the crush-injured facial nerve (Tanzer & Jones, 1997). In the present study, using a model of ISCI, we did not address the direct effects of sex hormones on axonal regeneration. It is possible that testosterone has opposing effects after nerve injury; a direct effect, in which the regenerative properties of motor neurons are enhanced via molecular mechanisms that involve selective alterations of the neuronal cytoskeleton (Jones *et al.*, 1999), and an indirect effect on autoimmune neuroprotection through the suppression of cell-mediated immunity.

On the basis of the present findings, we suggest a mechanism in which the immunosuppressive effects of testosterone worsen the outcome of ISCI in males. As testosterone is the substrate for the synthesis of the active sex steroids DHT and E2, which have high binding affinities for androgen and oestrogen receptors, respectively (Balthazart, 1998), assigning any physiological function to testosterone is not a simple task. Evidence for the presence of androgen and oestrogen receptors in the rat spinal cord was provided by MacLusky *et al.* (1987), whose failure to detect aromatase activity in the spinal cord is consistent with their hypothesis that the effects of circulating testosterone on spinal reflex function are mediated primarily through the androgen receptor system (MacLusky *et al.*, 1987). The suggested effect of testosterone on T cell-mediated neuroprotection might be manifested in the periphery. After complete spinal cord transection, serum testosterone in adult male rats is suppressed (Huang *et al.*, 1995; Huang *et al.*, 1999), possibly as a result of denervation of the testicles. However, testosterone suppresses humoral and cell-mediated immune responses at all concentrations (Paavonen *et al.*, 1981; Sthoeger *et al.*, 1988; Kovacs *et al.*, 2002). Female mice with testosterone implants display significantly lower cell-mediated immune responses *in vivo* than nonimplanted controls and testosterone receptor blockade was found to restore cellular immunity in male mice (Angele *et al.*, 1998; Weinstein *et al.*, 1984; Messingham *et al.*, 2001). In the present study, female rats and mice in different experimental settings, regardless of their estrous cycle-dependent E2 levels, showed significantly better functional and morphological outcome than their male littermates (Figs 1–4 and 7). Also, the androgen receptor antagonist flutamide significantly improved the functional outcome of ISCI in males (Fig. 6C and D). These findings suggest that testosterone, through the effect of its androgen receptor on immune related activities, has an adverse effect on recovery from ISCI.

It thus seems from the present study that the very factor that appears to give females an advantage over males in responding to a CNS insult, namely, the ability (which in males is relatively suppressed) to sustain an autoimmune response, places them at a disadvantage with respect to autoimmune diseases, unless they are constitutionally endowed with a naturally occurring immune regulatory mechanism (Kuchroo *et al.*, 2002; Singh *et al.*, 2001). It seems, however, that autoimmunity is regulated on at least two levels: (i) the overall ability of the individual to sustain an autoimmune response, and (ii) the genetically determined regulation of this autoimmune response in such a way as to derive the benefit of protection without the risk of autoimmune disease. The activities of these two control mechanisms would lead, in general, to a better recovery from injury in females than in males of any strain, and to a higher incidence of autoimmune disease in females than in males among individuals of EAE-susceptible strains. Accordingly, females of resistant strains may enjoy the benefit of protective autoimmunity without paying the price of incurring an autoimmune disease, whereas males of susceptible strains may suffer both the harmful effects of autoimmunity (though less frequently than susceptible females) and poor recovery from traumatic injury. We suggest that manipulation of the immunomodulatory properties of sex hormones, for example by preventing testosterone-related post-traumatic immunodepression, might represent a novel therapeutic strategy for the treatment of traumatic injuries.

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Abbreviations

CNS, central nervous system; DHT, dihydrotestosterone; EAE, experimental autoimmune encephalomyelitis; ISCI, incomplete spinal cord injury; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus; WT, wild-type.

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